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14. ABSTRACT
The objective of this proposal is to test targeted carbon nanotubes for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) are efficient transducers of near-infrared radiation (NIR) for laser-induced thermal therapy of kidney cancer in mouse models. Our goal is to improve the anti-tumor efficacy of CNTs by designing them to target cancer cells and surrounding endothelial cells following systemic administration. Specifically, we will develop carbon nanotubes that bind to uPAR, a surface receptor overexpressed in kidney cancers and supporting endothelium. We will use D5, a peptide designed in the laboratory, as the targeting ligand. In the past year, we developed a new chemical approach to conjugating the targeting peptide to nanotubes. We demonstrated that the peptide is cytotoxic to kidney cancer cells. We also showed that the combination of nanotubes and NIR is effective in inhibiting the clonogenic survival of cultured kidney cancer cells. Next year, we will assess the flow of nanotubes in the vasculature and their ability to accumulate and exert an anti-tumor effect in a mouse tumor model. This grant is a mentor/predoctoral award that also focuses on training of a predoctoral candidate. The predoctoral fellow carried out the experiments described in this progress report, attended the national AACR cancer meeting, presented his work in seminars, and was co-first author on an article on nanotubes as thermal ablation agents.

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INTRODUCTION

The overall goal of this proposal is to test targeted carbon nanotubes for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) have been shown to be efficient transducers of near-infrared radiation for laser-induced thermal therapy of kidney cancer in animal models. However, the current generation of carbon nanotubes lacks the ability to selectively target cancer cells following systemic administration. In this proposal, we will develop carbon nanotubes designed to bind to uPAR, a surface receptor overexpressed in kidney cancers that is involved in growth, migration, proliferation, metastasis and angiogenesis. Binding of peptide fragments of kininogen (D5) to uPAR induces apoptosis in endothelial cells and inhibits tumor growth and metastasis. We will combine the anti-tumor properties of CNTs with those of D5 into one combined treatment by conjugating D5 peptides to several types of multiwall carbon nanotubes. *In vitro* experiments will be performed to test the specificity of binding of these conjugates to uPAR in human endothelial cells, kidney cancer cells, and kidney cells. Thermoablative properties, proliferation, survival, apoptosis, and downstream signaling of uPAR will be examined in these cell lines following treatment with D5 nanotubes. The anti-tumorigenic effect of these nanotubes will also be studied *in vivo*. Biodistribution, accumulation, and thermoablation will be studied initially. Human kidney cancer cells will be injected into the kidney capsule of nude mice. The effect of D5 nanotube injection and thermoablation on tumor growth and survival will be determined. The effect of D5 nanotubes on tumor angiogenesis will be studied by repeating these experiments using co-injection of human kidney cancer cells with human endothelial cells in a matrigel plug. We believe that these conjugated nanotubes will be able to demonstrate enhanced tumor ablation via targeting compared to thermal ablation alone.

BODY:

Specific Aim 1. Synthesize nanotube-based particles ligated to D5s.

Progress on the Tasks from the Statement of Work associated with accomplishing this Specific Aim is described below.

Task 1: Obtain linear, Y-branched and dendritic nanoparticles. We obtained the first batch of branched nanotubes in year 1 of the grant. In year 2, we obtained nanotube variants with precisely defined wall thicknesses from our collaborator at Rice, Dr. Ajayan. We have been testing whether thermal properties can be improved and controlled through the use of such variants.

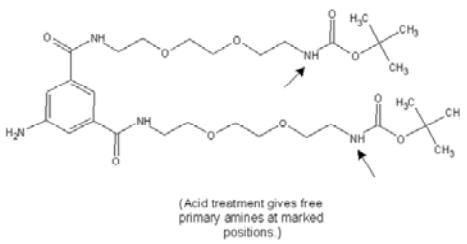

Figure 1. Linker for peptide conjugation

Task 2: conjugate linear, Y-branched and dendritic nanotubes to the synthetic peptide, D5s.

We are continuing to optimize the methodology for conjugation of peptides to nanotubes. This facet of the

project is led by Dr. King, a collaborator on this project. In particular, we have been developing a new "linker" compound for amidation with COOH defect groups on nanotubes. Our initial studies indicated that we needed a linker that is easy to prepare on a large scale, has good solubility in water, and has reactive functionality for further chemistry. We developed a fast and scalable synthesis of a linker from 5-nitroisophthalic acid, as shown in Figure 1. We conjugated this linker to nanotubes using amidation with EDAC, as shown in Figure 2. We plan to capitalize on this strategy to prepare D5-conjugated nanotubes in sufficient quantity to allow both detailed characterization and animal studies.

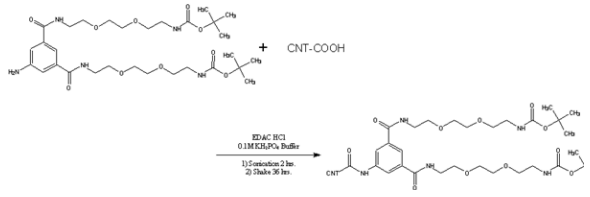

Figure 2. Conjugation of linker to nanotubes using EDAC-based chemistry.

Specific Aim 2. Test binding, cytotoxic and thermoablative properties of D5s-nanoparticles *in vitro*.

2.a. Test specificity of binding of D5a-nanoparticles.

Task 2:1-2.3: Confirm that binding occurs via specific binding to UPAR and test ability of D5s-nanoparticles to target proliferating endothelial cells and kidney cancer cells.

To confirm binding of D5 to uPAR, GST pulldown experiments were performed using cultured kidney cancer cells and GST-tagged D5. 500 ug of lysate was incubated with GST-D5 or GST and rotated overnight at 4^{o} C. The lysates were then incubated with GST-binding agarose beads, washed, eluted, and ran on SDS PAGE. Binding was detected by immunoblotting for GST and uPAR (**Figures 3** and **4**). Immunoblotting demonstrates that we can detect both GST and uPAR.

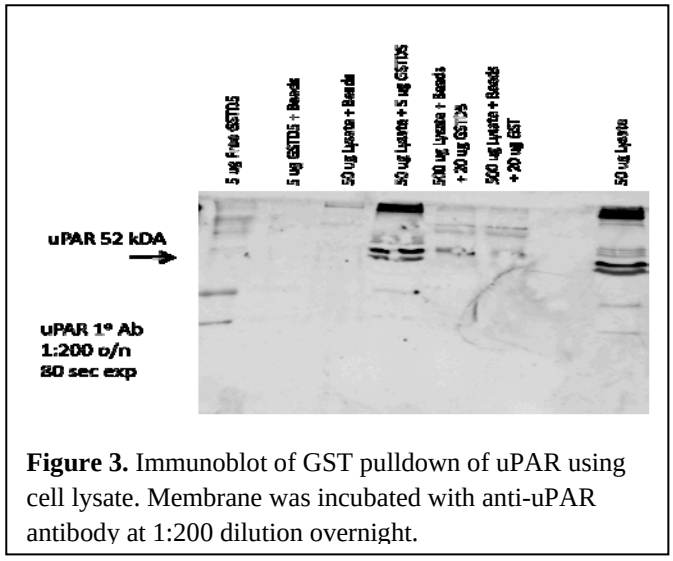

Figure 3. Immunoblot of GST pulldown of uPAR using cell lysate. Membrane was incubated with anti-uPAR antibody at 1:200 dilution overnight.

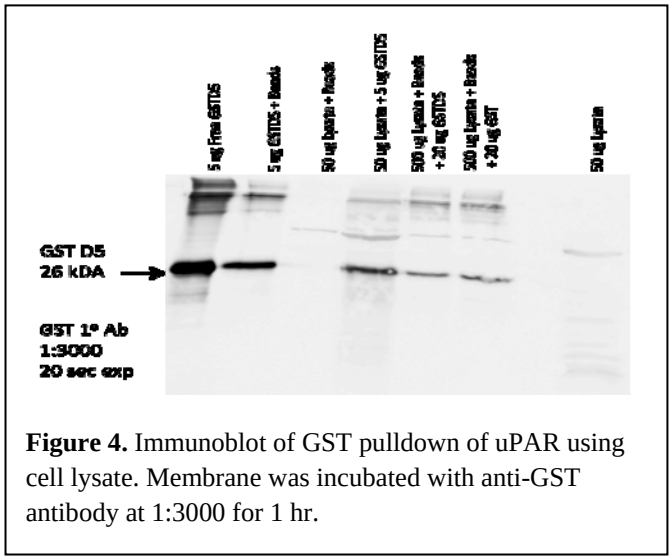

Figure 4. Immunoblot of GST pulldown of uPAR using cell lysate. Membrane was incubated with anti-GST antibody at 1:3000 for 1 hr.

2.b. Assess anti-proliferative effects of D5a-nanoparticles.

Task 2.4. Assess anti-proliferative effects of D5a-nanoparticles.

<u>D5 Toxicity</u>

We had previously shown that D5 was toxic to endothelial cells. Since our hypothesis is that D5

can simultaneously target proliferating endothelial cells and kidney cancer cells, we next assessed the anti-proliferative effects of D5 in kidney cancer cells. 6000 CRL1932 kidney cancer cells were plated, starved and incubated with recombinant D5, HKa (cleaved high-molecular-weight kininogen), or staurosporine (positive control) for 28 or 48 hrs. 5 wells were used for each treatment group. Dose-dependent decreases in viability (determined by MTT assay) were seen in CRL1932 cells incubated with D5 (**Figures 5** and **6**). Upon 24 hr. incubation, we found ~50% growth inhibition in the CRL1932 cell line at 200 nM HKa and at 500 nM D5. At 48 hr. incubation, 50% growth inhibition was achieved with 100 nM D5 treatment and 25% inhibition at 150 nM D5. We have previously shown similar 90% growth inhibition of HUVECs incubated with 50 nM D5 for 24 hrs. These results indicate that D5 possesses the desired cytotoxic effect on kidney cancer cells.

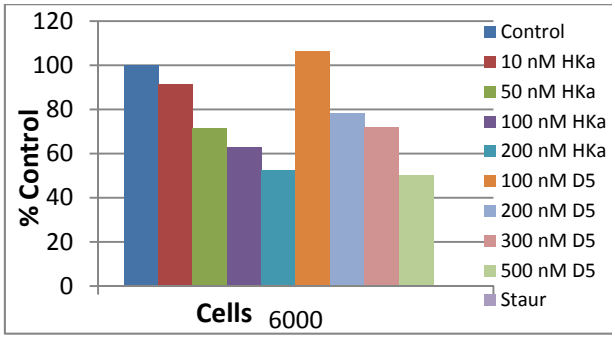

Figure 5. MTT assay of CRL1932 incubated with HKa or D5 for 24 hrs

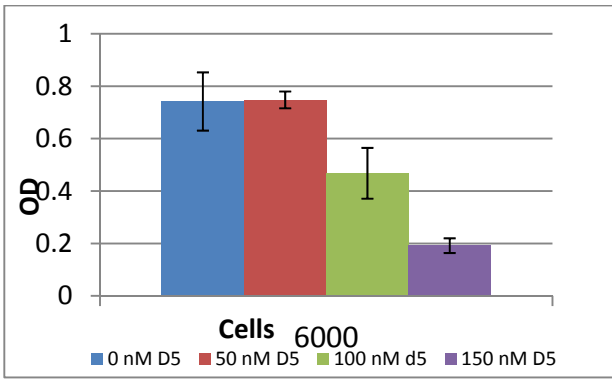

Figure 6. MTT assay of CRL1932 cells incubated with D5 for 48 hrs.

<u>CNT Toxicity</u>

To measure the inherent toxicity of D5-conjugated CNTs, we also tested the cytotoxicity of CNTs prior to conjugation. For these experiments we used RENCA kidney cancer cells incubated with increasing concentrations of pristine (unmodified), carboxylated (COOH functionalized), and amidated (NH2 functionalized) CNTs for 24 hrs. Clonogenic survival was assessed 7-10 days after CNT incubation after plating 200-300 cells/well (**Figure 7**). Similar trends were seen using all three types of CNTs in the two cell lines studied, and demonstrated that nanotubes alone exhibit mild toxicity (Figure 7).

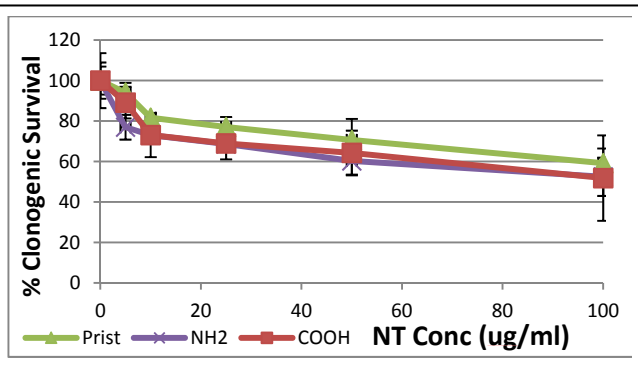

Figure 7. Clonogenic survival of RENCA cells treated with increasing concentrations of MWCNTs.

2.c. Assess thermal ablative properties of D5a-nanoparticles.

Task 2.5. Assess thermal ablative properties of D5a-nanoparticles.

<u>Cell Survival upon Heating</u>

To assess the effect of CNT heating on cell survival, RENCA and CRL1932 cell lines were heated for 30 or 45 sec. at 3 W laser power with a 1064 nm YAG laser. Experiments were

performed with 100 ug/ml pristine (unmodified), carboxylated (COOH functionalized), and amidated (NH2 functionalized) CNTs. After heating, cells were plated at 300 cells/well. In both cell lines, heating alone and CNT treatment alone results in ~10-30% cell killing, while heating with CNTs results in almost no surviving colonies. (**Figures 7** and **8**). Notably, the combination of nanotubes and near infrared-radiation led to a substantial decrease in survival in cells treated with amidated nanotubes (Figure 8). This is very promising, since these model the D5-nanotubes we will be using in future experiments. Further, CNT toxicity to RENCA mouse kidney cancer cells was very close to that observed with human kidney cancer cells or endothelial cells, suggesting that it will be possible to perform these experiments in vivo using a syngeneic mouse.

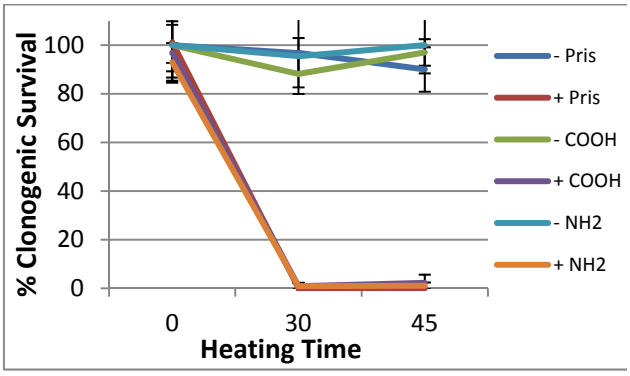

Figure 8. Clonogenic survival of RENCA cells after MWNT treatment and heating with 3 W for 30 or 45 sec.

Specific Aim 3. Examine accumulation and anti-tumor effect of particles in mice.

Work on Aim 3 will be carried out in the upcoming grant year. In preparation for these studies, we have worked on detection of unmodified nanotubes in tumor sections.

KEY RESEARCH ACCOMPLISHMENTS:

- Tested a new chemical strategy for large-scale production of soluble D5-conjugated nanotubes
- Demonstrated modest toxicity of unconjugated nanotubes to kidney cancer cells
- Demonstrated that the combination of NIR and nanotubes can successfully inhibit the survival of both human and mouse kidney cancer cells.

REPORTABLE OUTCOMES: The trainee, Peter Alexander, has attended meetings, given presentations, and written a manuscript to enhance his training with regard to nanotechnology and use of nanotechnology in anti-cancer therapy. In particular, he attended the national meeting of the American Association for Cancer Research. He formally presented his research in a seminar at Wake Forest University. In addition he was co-first author of a review on the use of carbon nanotubes in thermal therapy. Writing this review not only familiarized him with the literature, but honed his writing skills. This review is currently in press:

Ravi N. Singh*, Peter Alexander*, Andrew R. Burke, Frank M. Torti, Suzy V. TortiCarbon Nanotubes for Thermal Therapy. *In* Cancer Nanotechnology: Principles and Applications in Radiation Oncology. Edited by Sang Hyun Cho, Ph.D., Georgia Institute of Technology and Sunil Krishnan, M.D., The University of Texas M. D. Anderson Cancer Center. In press, 2012.

*These authors contributed equally to this chapter

CONCLUSION:

In the past grant year, we made good progress towards the goal of testing the anti-tumor effect of D5-nanotubes in a variety of in vitro contexts. In the spring of 2012 I moved my laboratory to the University of Connecticut, where a new student, Erik Carboni, will be continuing this project. In the upcoming year we hope to test transit properties of nanotubes in model vasculature and the effect of D5 conjugation on these properties. We also plan to test anti-tumor efficacy in an in vivo model.

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